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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

PALLIARD

Serial No.: 09/894,845

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Group Art Unit: 1635

Examiner: J. Angell

Title: TOLERANCE AND CHRONIC HEPATITIS C VIRUS

DECLARATION OF MICHAEL HOUGHTON, Ph.D.

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

I, Michael Houghton, hereby declare as follows:

1. I received my Bachelors of Science Degree in the Biological Sciences from the University of East Anglia at Norwich, England in 1972, and my Doctor of Philosophy Degree in Biochemistry from King's College, University of London in 1977.

2. I have been employed by Epiphany Biosciences, Inc. since 2007 and currently hold the position of Chief Scientific Officer. I previously served as Vice-President of Hepatitis C and Virology Research at Chiron Corporation and Novartis Vaccines & Diagnostics, Inc., which acquired the technology that is the subject of this application. I am currently a paid consultant for Novartis Vaccines & Diagnostics, Inc. I am extremely familiar with hepatitis C virus (HCV) and, in particular, with HCV pathogenesis, including the development of immune tolerance in infected patients and liver pathology associated with acute and chronic HCV infection, having actively studied and worked in this discipline for over 25 years. I have coauthored numerous publications and patents relating to HCV immunopathology. A copy of my Curriculum Vitae is attached hereto as Exhibit A.

3. I have reviewed relevant documents from the prosecution of the above-referenced application (hereinafter "the application"), including the Office Action dated April 18, 2007 and

the art cited therein, the Advisory Action dated July 9, 2007, and the claims as amended October 18, 2007. I understand the claims have been rejected over the reference of Gorczynski et al. (Cellular Immunol. (1995) 160:224-231; hereinafter "Gorczynski") in view of Nakai et al. (Blood (1998) 91:4600-4607; hereinafter "Nakai"), and further in view of Wakita et al. (J. Biol. Chem. (1998) 273:9001-9006; hereinafter "Wakita") and Donnelly et al. (WO 97/47358; hereinafter Donnelly).

4. I understand that the claims pending in the application are directed in part to methods of preparing a non-human animal for screening for agents that modulate tolerance to a hepatitis C virus (HCV) immunogen by preparing a nucleic acid directing liver-specific expression of an HCV immunogen, and exogenously delivering the nucleic acid to the liver of the animal by portal vein injection under conditions that result in the sustained expression of the HCV immunogen for at least one month in the liver thereby inducing immunological tolerance to the HCV immunogen.

5. I understand that the claims pending in the application have been rejected in whole or in part based on the teachings of Gorczynski, Nakai, Wakita, and Donnelly. I also understand that the Examiner is of the opinion that it would have been obvious to combine the teachings of these four references, at least in part due to the fact that a person of ordinary skill in the art would have had a reasonable expectation of success in making an animal tolerant to an HCV antigen given that portal vein injection of non-HCV antigens has been used for inducing tolerance to other immunogens.

6. I do not agree the combination of references cited in the Office Action describe the invention claimed or render the claimed invention obvious, *i.e.*, that the differences between the invention as claimed and the subject matter of the cited art are such that they would be obvious to one skilled in the art, such as myself, as of the filing date of the patent application or earlier. My opinion is based on the facts set forth below and my familiarity with the subject matter. In particular, I disagree with the Examiner that one of skill in the art would be motivated to combine the teachings of Gorczynski with those of Nakai, Wakita, and Donnelly, as Gorczynski describes the injection of lymphoid or spleen cells into the portal veins of mice a few

days before skin graft transplants to delay transplant rejection and fails to describe anything pertaining to HCV.

7. There remains a need in the art to find suitable animal models for screening for agents that can modulate or reverse immunological tolerance to HCV antigens. None of the cited references describe an animal model of immunological tolerance in which antigens are specifically targeted and restricted to the liver. HCV replication occurs almost exclusively in the liver where tolerance to the virus develops due to the specialized liver environment, which limits T cell activation and function. See, e.g., Crispe, IN, *Nat. Rev. Immunol.* (2003) 3:51-62, attached as Exhibit B. The use of liver-specific promoters and enhancers in the animal model of the instant invention restricts expression of HCV antigens to the liver, and therefore more accurately mimics the natural development of tolerance to HCV immunogens in the liver during viral infection.

8. Unlike the animal model of the instant invention, expression of HCV antigens in the transgenic animal model described by Wakita is not liver-specific. Genes encoding HCV antigens are present in every tissue of the transgenic mice and are expressed using a CAG promoter that is not liver-specific (page 9002, col. 1). Furthermore, the adenovirus vector encoding the Cre trans gene, which is used to turn on expression of genes encoding HCV antigens, though injected into the tail vein to target the liver, is not restricted to the liver. In fact, expression of HCV antigens is detected in a variety of tissues outside of the liver in Wakita's transgenic mice, including in the lung, spleen, thymus, kidney, stomach, intestines, and muscles (see page 9004, col. 1). Moreover, in the CN2-29 transgenic mice described by Wakita, the expression of the HCV core antigen is at about the same level in the spleen (5.5 ng/mg) as in the liver (6.6 ng/mg) and only about 2-fold less in the lung (2.9 ng/mg). Thus, the transgenic mice of Wakita express HCV antigens in multiple tissues where immunoreactivity is not dampened in the same way as the liver.

9. Similarly, Gorczynski fails to describe or suggest methods for limiting exposure of antigens to the liver of animals. Gorczynski describes injection of lymphoid or spleen cells into the portal vein of mice; however, the cellular antigens are not restricted to the liver and can

migrate elsewhere. Thus, both Gorczynski and Wakita describe animal models in which antigens are exposed to the host's immune system outside of the tolerogenic environment of the liver. The instant invention, in contrast, uses liver-specific promoters and enhancers (*e.g.*, alpha-1 anti-trypsin (AAT) promoter and apolipoprotein E (ApoE) enhancer) to ensure that expression of HCV antigens is restricted to the liver, better mimicking the natural biology of the virus and the evolution of tolerance.

10. The Wakita transgenic animal model also has a number of additional drawbacks. For one, transgenic animals are usually less desirable as models of tolerance because of the presence of antigens at birth. The immune system views antigens present at birth as "self" antigens and produces long term immunological tolerance to self-antigens by thymic deletion of T cells specifically immunoreactive with those antigens. In contrast, the later development of tolerance to non-self antigens by exposure of antigens in the liver has a different underlying mechanism. Therefore, animal models in which antigens are expressed at birth do not provide a good model of tolerance to non-self antigens as develops from exposure of antigens in the liver later in life. See, e.g., Waddington et al., *Curr. Opin. Mol. Ther.* (2007) 9:432-438, attached as Exhibit C.

11. Although, Wakita uses the Cre/loxP system for conditional expression of HCV antigens in the transgenic mice, genes encoding the HCV antigens are present at birth in every tissue of the animal and may be expressed at some basal level in the absence of Cre/loxP. For that matter, Wakita observes detectable levels of expression of the HCV core protein in the lung, spleen, thymus, kidney, stomach, intestines, and muscle even though Cre-mediated transgene recombination occurs only in the liver (see page 9004, col. 1). Hence, in these transgenic animals, the immune system may be exposed to HCV antigens due to leaky expression of viral genes, albeit at a low level, from birth. In contrast, the instant application provides a non-germline animal model of tolerance that more accurately mimics the natural development of tolerance during chronic HCV infection.

12. In addition, it is much more expensive and time consuming to produce numerous transgenic animals, as described by Wakita, for screening for agents that modulate

immunological tolerance. The use of portal vein injection of nucleic acids encoding HCV immunogens, as described in the present application, greatly facilitates screening. One of skill in the art can quickly design, for example, a dozen vectors encoding different HCV epitopes and inject such vectors into the portal vein of an animal to test for the development of immunological tolerance and screen for modulators that reverse tolerance. For example, various immunodominant HCV epitopes can be rapidly screened by this method for the development of immunological tolerance and agents that relieve tolerance. Thus, this method greatly increases the ease and flexibility of screening. In contrast, producing a dozen or more transgenic animals, as described by Wakita, to test the same epitopes would entail a great deal more effort, expense, and time.

13. Based on the foregoing, namely that the combination of Gorczynski, Nakai, Wakita, and Donnelly fails to teach or suggest the method of the claimed invention, including in particular, the injection of a nucleic acid directing liver-specific expression of an HCV immunogen into the portal vein of an animal, I do not agree that the claimed invention is obvious in view of the cited references.

14. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 26th October, 2007 Signature: M Houghton

Michael Houghton, Ph.D.

CURRICULUM VITAE

MICHAEL HOUGHTON

Education

1969-1972	*B.Sc. (Honors) Biological Sciences* *University of East Anglia,* *Norwich, England*
1973-1977	*Ph.D. Biochemistry* *King's College,* *University of London,* *England*

Positions

1977 – 1982	*Senior Research Investigator, Human interferon genetics, Searle Research Laboratories, Buckinghamshire, England*
1982 – 1988	*Project Leader, Non-A,Non-B Hepatitis Discovery Research, Chiron Corporation, Emeryville, CA*
1988 – 2000	*Director, Hepatitis C Research, Chiron Corporation, Emeryville, CA*
2000 – 2003	*Vice-President, Hepatitis C Research, Chiron Corporation, Emeryville, CA*
2003 – 2006	*Vice-President, Hepatitis C & Virology Research, Chiron Corporation, Emeryville, CA*
2006 – 2007	*Vice-President, Hepatitis C & Virology Research, Novartis Vaccines & Diagnostics, Inc., Emeryville, CA*
2007 -	*Chief Scientific Officer, Epiphany Biosciences Inc., San Francisco, CA*

Awards

The following honors were awarded for research on hepatitis C:

1. *Co-recipient of the Karl Landsteiner Award from the American Association of Blood Banks (1992)*
2. *Co-recipient of the Robert Koch Award from Germany (1993)*
3. *Honoree of the Japanese Medical Congress (1993)*
4. *Honoree of the Triennial International Hepatitis Meeting (1993)*
5. *Co-recipient of the William Beaumont Prize from the American Gastroenterology Association (1994)*
6. *Recipient of Beatrice Bitiello Award from Italian Association for Prevention of Viral Hepatitis (1994)*
7. *Awardee of the Princess Takamatsu Cancer Research Fund(1994)*

8. *Co-recipient of the International Hepatitis Foundation Award (1998)*
9. *Co-recipient of the Hans Popper Award (Falk Foundation;1999)*
10. *Co-recipient of the Clinical Lasker Award (2000)*
11. *Co-recipient of the Dale Smith Memorial Award of the American Association of Blood Banks*

Patents

Numerous patents issued in the fields of recombinant human interferons, bacterial expression vectors, Hepatitis C and D viruses.

Publications

Transcriptional and translational control in eukaryotes

1. *"The purification and properties of hen oviduct Form B DNA-dependent RNA polymerase" M. Houghton and R.F. Cox (1974) Nucl. Acids Res. 1, 299-308.*
2. *"The presence of ovalbumin mRNA coding sequences in multiple restriction fragments of chicken DNA" M.T. Doel, M. Houghton, E.A. Cook and N.H. Carey (1977) Nucl. Acids. Res. 4, 3701-3713.*
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6. *"Active multi-subunit ACh receptor assembled by translation of heterologous mRNA in Xenopus oocytes." K. Sumikawa, M. Houghton, J.S. Emtage, B.M. Richards and E.A. Barnard (1981) Nature 292, 862-864.*
7. *"The molecular cloning and characterization of cDNA coding for the a subunit of the acetylcholine receptor." K. Sumikawa, M. Houghton, J.C. Smith, L. Bell, B.M. Richards and E.A. Barnard (1982) Nucl. Acids Res. 10, 5809-5822.*
8. *"Cloning and sequence determination of the gene for the human $\sum$ immunoglobulin & chain expressed in a myeloma cell line." J.H. Kenten, H.V. Molgaard, M. Houghton, R.B. Derbyshire, J. Viney, L.O. Bell and H.J. Gould (1982) P.N.A.S. (USA) 79, 6661-6665.*

9. *"A study of the mRNA and genes coding for the nicotinic acetylcholine receptor." K. Sumikawa, M. Houghton, R. Miledi and E.A. Barnard (1983) in "Cell Surface Receptors," Ed. P.G. Strange pp. 249-269 (Ellis Horwood Ltd., U.K.).*

10. *"Molecular genetics of the acetyl choline receptor and its insertion and organization in the membrane", E.A. Barnard, M. Houghton, R. Miledi, B.M. Richards, and K. Sumikawa, Biol. Cell (1982) 45:383.*

Molecular genetics of human fibroblast interferon

11. *"The amino-terminal sequence of human fibroblast interferon as deduced primers." M. Houghton, A.G. Stewart, S.M. Doel, J.S. Emtage, M.A.W. Eaton, J.C. Smith, T.P. Patel, H.M. Lewis, A.G. Porter, J.R. Birch, T. Cartwright and N.H. Carey (1980) Nucl. Acids Res. 8, 1913-1931.*

12. *"Human interferon gene sequences" M. Houghton (1980) Nature 285, 536.*

13. *"The complete amino acid sequence of human fibroblast interferon as deduced using synthetic oligodeoxyribonucleotide primers of reverse transcriptase" M. Houghton, M.A.W. Eaton, A.G. Stewart, J.C. Smith, S.M. Doel, G.H. Catlin, H.M. Lewis, T.P. Patel, J.S. Emtage, N.H. Carey and A.G. Porter (1980) Nucl. Acids Res. 8, 2885-2894.*

14. *"The absence of introns within a human fibroblast interferon gene" M. Houghton, I.J. Jackson, A.G. Porter, S.M. Doel, G.H. Catlin, C. Barber and N.H. Carey (1981) Nucl. Acids Res. 9, 247-266.*

15. *"The cloning and expression of a human fibroblast interferon gene in bacteria." M. Houghton, S.M. Doel, G.H. Catlin, A.G. Stewart, A.G. Porter, W.C.A. Tacon, M.A.W. Eaton, J.S. Emtage and N.H. Carey (1981) Proceedings of the Battelle International genetic Engineering Conference, M. Keenberg, Ed. Battelle Seminars and Studies Program.*

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33. *"Prevalence of antibodies to hepatitis C virus in Italian patients with hepatocellular carcinoma", M. Colombo, Q.-L. Choo, E. Del Ninno, N. Dioguardi, G. Kjo, M.F., Donato, M.A., Tommasini and M. Houghton, The Lancet, (1989) p. 1006-1008.*

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35. *"A cDNA fragment of hepatitis C virus isolated from an implicated donor of post-transfusion non-A, non-B hepatitis in Japan. K. Takeuchi, S. Boonmar, T. Katayama, Q.L. Choo, G. Kuo, A.J. Weiner, D.W. Bradley, M. Houghton, I. Saito, T. Miyamura. Nucl. Acids Res. (1989) 17, 24:10367-10372.*

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44. *"Hepatitis C HCV associated idiopathic chronic hepatitis and cryptogenic cirrhosis", L. Jeffers, M. de Medina, F. Hasan, R. Reddy, T. Parker, M. Silva, L. Mendez, E. Schiff, M. Houghton, Q.-L. Choo and G. Kuo, Hepatology (1989) 10:644.*

45. *"Hepatitis C HCV associated hepatocellular carcinoma", F. Hasan, L. Jeffers, M. de Medina, R. Reddy, T. Parker, E. Schiff, M. Houghton, Q.-L. Choo, and G. Kuo, Hepatology (1989) 10:608.*

46. *"Seroepidemiology of hepatitis C virus HCV in selected population", A.A. Evans, H. Cody, G. Kuo, Q.-L. Choo, M. Houghton, W.N. Katkov and J.L. Dienstag, Hepatology (1989) 10:644.*

47. *"Blood-borne non-A, non-B hepatitis PT-NANB immunohistochemical identification of disease and hepatitis C virus-associated antigens", K. Krawczynski, G. Kuo, A. Dibisceglie, M. Houghton and D.W. Bradley, Hepatology (1989) 10:580.*

48. *"Detection of antibody to hepatitis C virus in patients with various chronic liver diseases", A.M. De Bisceglie, H. Alter, G. Kuo, M. Houghton and J.H. Hoofnagle, Hepatology (1989) 10:581.*

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HEPATIC T CELLS AND LIVER TOLERANCE

Ian Nicholas Crispe

The T-cell biology of the liver is unlike that of any other organ. The local lymphocyte population is enriched in natural killer (NK) and NKT cells, which might have crucial roles in the recruitment of circulating T cells. A large macrophage population and the efficient trafficking of dendritic cells from sinusoidal blood to lymph promote antigen trapping and T-cell priming, but the local presentation of antigen causes T-cell inactivation, tolerance and apoptosis. These local mechanisms might result from the need to maintain immunological silence to harmless antigenic material in food. The overall bias of intrahepatic T-cell responses towards tolerance might account for the survival of liver allografts and for the persistence of some liver pathogens.

SINUSOID
A blood-filled space that lacks the anatomy of a capillary. Sinusoids generally contain slow-flowing blood, which facilitates cellular interactions. Such vessels are found in the bone marrow and in the liver.

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The liver is an organ in which blood from the intestines, which is rich in bacterial products and in mainly harmless food-derived antigens, interacts with the circulating T-cell pool. The constitutive presence of non-self and microbial molecules imposes constraints on immune responses that are generated in the liver, and there might be distinctive control mechanisms that determine whether antigen encounter will result in immunity or tolerance.

Reasons to explain why the liver is often a site of immune tolerance have not been established, but one reasonable speculation is that it is because the liver is a site where harmless food antigens from the gut are processed and presented to the immune system. However, the liver is also subjected to invasion by pathogens that breach the intestinal mucosa and invade the circulation. Immune tolerance towards such invaders would not be advantageous, which indicates that liver lymphocytes must be able to switch rapidly from a tolerant to a responsive state.

Liver dendritic cells (DCs) facilitate the priming of T cells, and virus infections of the liver can generate T-cell immunity that allows the infection to be cleared, as is generally the case for hepatitis A virus in healthy humans and mouse hepatitis virus in immunocompetent mice[1–3]. However, several infections of the liver persist despite the development of an immune response, including three that are of great epidemiological significance: malaria, hepatitis B virus (HBV) and hepatitis C virus (HCV)[4–6] (BOX 1). Also, early in the history of experimental transplantation, immunologists were surprised to discover that in many species, allogeneic liver grafts can be established and maintained without immunosuppression[7]. By contrast, skin, kidney and other allografts are rejected rapidly. These results raise the question of whether there is a global phenomenon of 'liver tolerance'. In support of this concept, tumours that often metastasize to the liver include malignant melanoma, which expresses well-defined tumour antigens[8], as well as breast and lung cancers in humans. This indicates that the liver might be a site in which immunogenic tumour cells can evade immune surveillance.

To attempt to understand the control of tolerance and immunity in the liver, I discuss the anatomy of the liver blood vessels (SINUSOIDS), which facilitate immune-cell interactions. The large macrophage population of the liver is described briefly, as are the unusual lymphocyte populations that are present in this organ. However, these considerations do not provide an easy explanation for the baseline state of T-cell tolerance, because the lymphocyte populations that are unusually abundant in the liver have a well-defined role in promoting anti-pathogen immunity. The 'professional' antigen-presenting cells (APCs) of the liver include DCs and an unusual type of vascular endothelial cell,

POLYPROTEIN
A large protein that must be cleaved to yield many functional proteins. The ten proteins of hepatitis C virus are synthesized as a single polyprotein.

LIVER SINUSOIDAL ENDOTHELIAL CELLS (LSECs). These cells form the lining endothelium of the hepatic sinusoids. They have unusual morphology (with many small holes and no basement membrane) and unusual properties as antigen-presenting cells (a strong predisposition towards the induction of tolerance, despite the expression of many co-stimulatory molecules).

SIEVE PLATE
A cluster of small holes (fenestrae) in a liver sinusoidal endothelial cell, which is believed to facilitate diffusion between the hepatic sinusoid and the underlying space of Disse, which is where solutes can interact with hepatocytes.

KUPFFER CELLS
The macrophages of the liver. These cells are derived from blood monocytes, and they phagocytose particles, including bacteria, that enter the liver sinusoids.

LIVER SINUSOIDAL ENDOTHELIAL CELLS (LSECs). Among these cell populations, evidence has been found for antigen-presenting function that results in T-cell inactivation, both through the induction of apoptosis and through other mechanisms.

Anatomy, shaped by function

The liver stands between the gastrointestinal tract and the systemic circulation. Blood from the gut, which is rich in food antigens, environmental toxins and bacterial products, including endotoxin (lipopolysaccharide, LPS), is collected in the hepatic portal vein (FIG. 1). This vessel is the main source of blood flowing through the liver, but approximately 20% of the incoming blood arrives in the hepatic artery. These two blood supplies mix in the hepatic sinusoids, where the blood percolates from the portal tracts to the central veins, passing between plates of hepatocytes through spaces that are lined by LSECs (FIG. 2a). This organization maximizes the exchange of molecules between the sinusoidal space and hepatocytes, allowing the liver to carry out its functions of digestion, detoxification and synthesis of plasma proteins. The sinusoidal endothelium is fenestrated, and the presence of clusters of small holes (known as SIEVE PLATES) raises the possibility that cells in the sinusoidal space might make direct contact with underlying connective tissue and hepatocytes[9]. Blood plasma, lymphocytes and DC precursors pass from the sinusoids into a sub-endothelial space, known as the space of Disse. From this space, lymph is collected, and it flows through lymphatic vessels that run in the portal tracts to the draining lymph nodes (FIG. 2b). The combination of slow blood flow, fenestrated endothelium and lack of a discrete basement membrane distinguishes liver sinusoids from other vascular beds, and it might provide T cells that pass through the liver with unique access to tissue cells.

Kupffer cells

The liver contains a large population of resident macrophages, known as KUPFFER CELLS. These cells are derived from blood monocytes, and they are found mainly in the hepatic sinusoids. However, Kupffer cells can sometimes pass through the space of Disse and make direct contact with hepatocytes[10]. They are mobile and actively phagocytic, and they can be marked and observed *in vivo* through their capacity to endocytose fluorescent microspheres[11]. Similar to other macrophages, Kupffer cells can phagocytose apoptotic cells[12,13] and microorganisms[14–16]. The interactions between Kupffer cells and T cells have been analysed *in vitro* using purified Kupffer cells, and *in vivo* by depletion techniques. In some studies, Kupffer cells from various species have been shown to act as effective APCs, resulting in T-cell proliferation and cytokine synthesis[17,18]. However, Kupffer cells might be involved also in tolerance. *In vitro*, the synthesis of nitric oxide by Kupffer cells causes them to suppress T-cell activation[19]. *In vivo*, systemic immune tolerance occurs in response to alloantigenic leukocytes injected into the portal vein[20]; this form of tolerance depends on Kupffer cells, because it is impaired if the Kupffer cells are depleted by treatment with gadolinium chloride[21].

Intrahepatic lymphocytes

The liver contains an unusual population of resident lymphocytes, among which $CD8^+$ T cells usually outnumber $CD4^+$ T cells, and both natural killer (NK) and natural killer T (NKT) cells are enriched relative to their proportions in lymphoid tissues[22,23]. Most of the intrahepatic $CD8^+$ and $CD4^+$ T cells have an activated phenotype. So, human liver $CD8^+$ T cells express CD25 and CD69 (REF. 24). In mice, the liver can trap activated $CD8^+$ T cells preferentially in perfusion experiments[25]. The intrahepatic $CD4^+$ T cells of mice have a $CD45RB^{low}$ phenotype, and they synthesize both interferon-γ (IFN-γ) and interleukin-4 (IL-4)[26]. When such activated $CD4^+$ T cells are delivered by adoptive transfer, some survive in the liver for weeks. Among these cells, those with a T helper 1 (T_H1)-type effector function become non-functional, whereas T_H2-biased $CD4^+$ T cells sustain their function[27]. In addition, a subset of liver T cells express the CD45 isoform B220, which is expressed more commonly by B cells[28]. The expression of B220 by T cells is associated with apoptosis, both *in vitro*[29] and in the liver[30].

The NK cells of rodent livers were identified first as 'pit cells', which were defined as large granular lymphocytes with cytotoxic activity against classic NK-cell targets, such as YAC1 cells[31]. These NK cells have long been known to increase in number during infection of the liver[32]. They express germline-encoded activating and inhibitory receptors, and they are present at an unusually high frequency among resident liver lymphocytes. During experimental liver injury induced by the injection of

Box 1 | Immune evasion by hepatitis C virus

Hepatitis C virus (HCV) is a main cause of liver disease in humans. HCV is a single-stranded RNA virus, for which a 9.5-kb messenger RNA encodes a single POLYPROTEIN. This polyprotein is cleaved by a combination of cellular and HCV-encoded peptidases into ten fragments. The World Health Organisation estimates that 170 million people are infected with HCV worldwide, and almost 4 million individuals are infected in the United States[150].

HCV primes the T-cell system, and $CD4^+$ and $CD8^+$ T-cell responses can sometimes co-exist with persistent infection[151,152]. In both chimpanzees and humans, an early and diverse $CD4^+$ and $CD8^+$ T-cell response is associated with clearance of the virus[153,154]. Sequence analysis of HCV isolates shows that HCV generates escape mutations in both of the two susceptible species[155–157]. This is a classic type of immune evasion, which is also seen for other persistent viruses.

HCV might disable T cells by other mechanisms also. Some mutations create antagonistic peptides with the potential to inactivate T cells that are specific for the prototype HCV antigenic epitope[157,158]. Such antagonism might explain the long-term persistence of individual virus genotypes, even in the presence of T-cell priming. In addition, HCV-specific T cells might become dysfunctional, particularly with regard to the synthesis of inflammatory cytokines. This has been termed 'stunning'[159]. The mechanism is not known.

Two HCV structural proteins, core protein and E2, have immunosuppressive properties *in vitro*. HCV core protein suppresses T-cell activation by binding to the C1q receptor[160,161], and it might also promote CD95 (FAS)-induced apoptosis[162]. The HCV envelope protein E2 binds CD81 on natural killer (NK) cells and inhibits NK-cell function[163]. The interpretation of this finding is complicated, because transgenic mice that express HCV core, E1 and E2 proteins have no evidence of global immunosuppression[164].

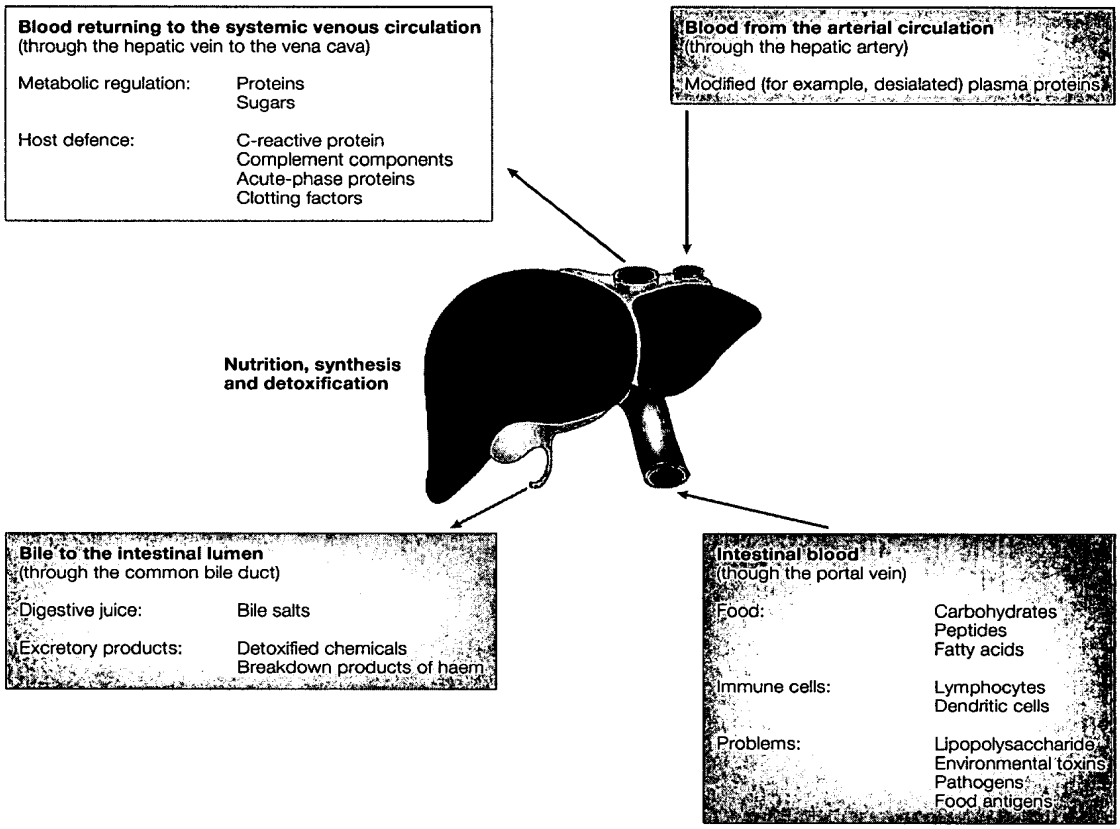

Figure 1 | **The inputs, outputs and main functions of the liver.** The figure shows that, in addition to its central metabolic role, and its interactions with T cells, which are summarized in this review, the liver makes an important contribution to host defence by synthesizing several defensive molecules, including complement components and clotting factors.

concanavalin A[33] and during infection of the liver with adenovirus vectors[34] (BOX 2), NK cells seem to have a crucial role in T-cell recruitment. During cytomegalovirus infection of mice, type 1 IFNs (IFN-α/β) induce synthesis of the chemokine CCL3 (also known as monocyte inflammatory protein-1α, MIP1α), which, in turn, is responsible for NK-cell accumulation[35]. Lack of CCL3 compromises both NK-cell accumulation and protective immunity. The NK cells synthesize IFN-γ, which promotes secretion of the chemokine CXCL9 (monokine induced by γ-interferon, Mig), probably by hepatocytes and/or LSECs[36], and this is responsible for the accumulation of T cells. Therefore, liver NK cells recruit T cells through a multi-step cytokine/chemokine cascade (FIG. 3), and in so doing, they promote T-cell immunity, rather than tolerance.

The normal mouse liver is also rich in NKT cells. These cells have a complex phenotype, features of which include the expression of NK1.1 in the B6 mouse and expression of the p70 chain of the IL-2 receptor (IL-2Rβ)[37]. The prototype NKT cells express, at intermediate density, a T-cell receptor (TCR)αβ with specificity for the MHC class-I-like molecule CD1d. Their TCR repertoire has limited diversity; the TCR β-chain is limited to Vβ8.2, Vβ7 or Vβ2, whereas the TCR α-chain is uniformly Vα14–Jα281, with a conserved junctional sequence[38,39]. These cells are present throughout the immune system, usually at a low frequency (<5% of all T cells), but they are abundant in the liver. A distinct, CD1d-independent population of NKT cells seems to be abundant at other tissue sites[40]. Conversely, there are CD1d-reactive T cells that do not use the Vα14–Jα281 receptor, but that might be involved in intrahepatic pathology[41]. Whereas the classical NKT cells originate in the thymus[42], an intrahepatic origin has been proposed for other NKT-like cells[43]. The lineage relationships of these other NKT-cell populations are not known, and their identification is complicated by the fact that conventional T cells can express NK-cell markers after activation[44,45].

The function of NKT cells is of great interest. Early on, NKT cells were shown to secrete IL-4, and they might, therefore, be seen as 'anti-inflammatory' lymphocytes[46]. In support of this idea, one well-documented function of these cells is protection from autoimmune disease in non-obese diabetic (NOD) mice, which lack these cells[47,48]. However, *in vivo* analysis indicates that, in the liver, NKT cells have a positive role in host defence. Mice lacking the CD1d molecule have been reported to lack defences against *Mycobacterium tuberculosis* and *Borrelia burgdorferi*[49,50]. The data on anti-protozoan immunity are conflicting, indicating an apparent role

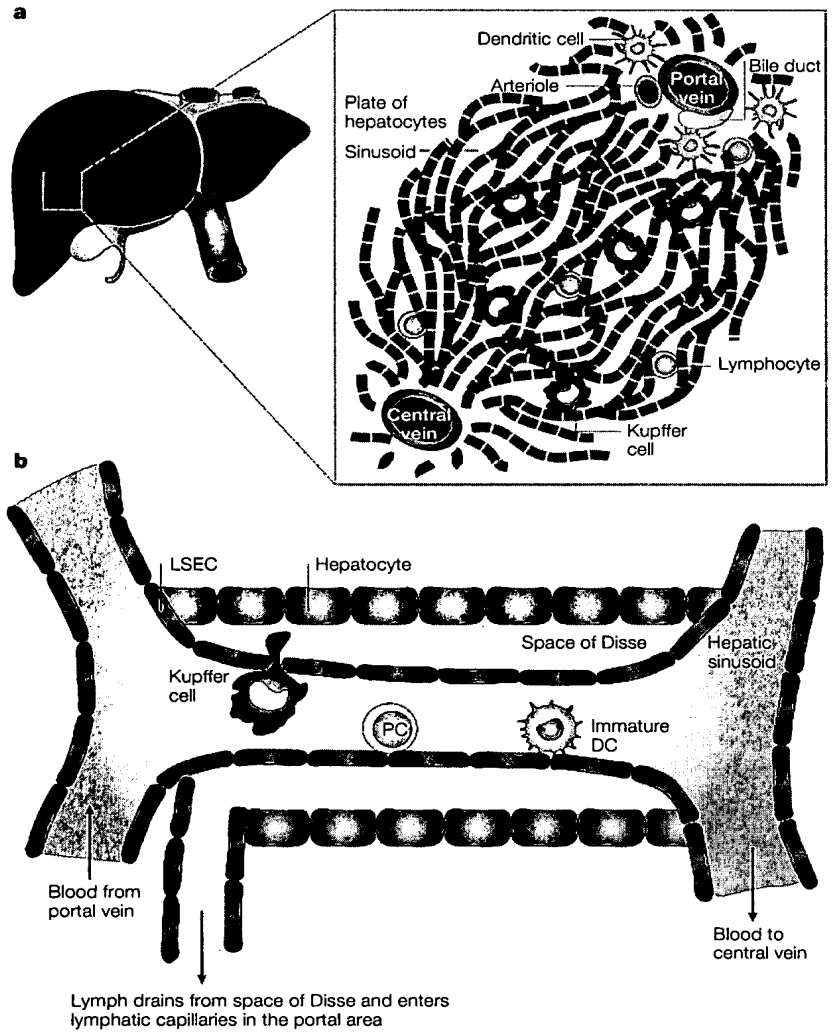

Figure 2 | **The hepatic microenvironment. a** | Diagram showing the structure of a liver lobule. The tissue is organized around vascular bundles, which are known as portal tracts. These contain a branch of the portal vein, an arteriole and a tributary of the bile duct. From the portal tracts, blood flows through a sponge-like anastamosing meshwork of sinusoids that exist between plates of hepatocytes. The sinusoids contain a large population of macrophages, known as Kupffer cells. **b** | Organization of sinusoids. The sinusoid is lined by an endothelium (liver sinusoidal endothelial cells, LSECs) that is fenestrated and lacks a basement membrane. Kupffer cells, lymphocytes (Pit cells, PCs) and immature dendritic cells (DCs) are found in the sinusoids. Kupffer cells exist mainly in the sinusoidal lumen, but they can make direct contact with hepatocytes. The sub-endothelial space, known as the space of Disse, is the region from which hepatic lymph originates.

for CD1d-reactive NKT cells in immunity to trypanosomes[51,52], but no role in immunity to mouse malaria[53]. One complication in the interpretation of these experiments is that the lack of CD1d might cause effects that are not due simply to the lack of NKT cells[54]. Mice that selectively lack the Vα14–Jα281 subset of NKT cells have been created, and these mice are deficient in anti-tumour immunity, which indicates that classical NKT cells have an important role in this process[55,56]. Another tool to study the function of NKT cells is α-galactosyl ceramide (α-GalCer), a molecule derived from marine sponges that causes acute NKT-cell activation, resulting in the synthesis of IFN-γ and IL-4, followed by deletion of the cells[57]. Using α-GalCer, the activation of NKT cells *in vivo* was accompanied by the inhibition of melanoma metastases[58], induction of effective immunity to the liver stages of malaria[59] and suppression of viral-RNA synthesis in HBV-transgenic mice[60]. In summary, there is strong evidence that liver NKT cells promote immunity, but no evidence to implicate these cells in liver tolerance.

The normal resident lymphocytes of the human liver have not been characterized in such detail, but similar to rodent liver lymphocytes, there is an increase in $CD8^+$ T cells relative to $CD4^+$ T cells, and an increase in the number of $CD3^+$ cells that co-express the NK-cell marker CD56 (REF. 22). Some, but not all, of these cells express a TCR in which the Vα24 segment is joined to JαQ, forming a conserved TCR that is analogous to the Vα14–Jα281 receptor on mouse NKT cells[61]. As in the mouse, the caveat must apply that the expression of NK-cell markers by αβ T cells could simply identify activated cells. A subset of normal human liver lymphocytes that express the markers CD2 and CD7 also express messenger RNA encoding recombination-activating gene 1 (RAG1), RAG2 and pre-Tα, which indicates that these cells might be undergoing lymphocyte-receptor gene rearrangement, and which supports the concept that the liver is a site of lymphoid development[62]. However, it is also possible that these mRNAs are expressed aberrantly by haematopoietic stem cells, which are present in human liver[63].

During inflammation, the lymphocyte populations of the liver change. In mice, experimental infection with *Propionibacterium acnes* causes a relative increase in the number of T cells and a corresponding decrease in the number of NKT cells[64], and the same changes are seen in mice with fatty livers[65]. Both of these pathologies sensitize the liver to endotoxin-mediated damage, which indicates that NKT cells might protect against such damage. In line with a tissue-sustaining function of NKT cells, the number of these cells increases during liver regeneration after partial hepatectomy[66]. Infection with lymphocytic choriomeningitis virus results in two phases of T-cell infiltration, involving heavy $CD8^+$ T-cell infiltration of the sinusoids, followed by $CD4^+$ T-cell infiltration of the portal tracts[67]. In humans, liver biopsy material provides a picture of liver lymphocytes during hepatitis. There are increases in the number of activated $CD8^+$ T cells[68] and in the number of $CD4^+$ T cells relative to the number of $CD8^+$ T cells[69]. During infection with HCV, the $CD8^+$ T cells are activated (they express CD45RO) and are located mainly in the sinusoids, whereas the $CD4^+$ T cells are mainly naive (they express CD45RA) and are located in the portal tracts[70]. The number of T cells that express the γδ TCR is also increased during viral hepatitis[71]. Overall, the inflamed liver shows a shift from the resting pattern of abundant NKT cells and an excess of $CD8^+$ T cells over $CD4^+$ T cells towards a more conventional pattern that is characteristic of other inflammatory sites. This is entirely consistent with a role for NK cells in initiating intrahepatic immune responses, but with their numbers then being diluted by the T cells that they recruit.

Interaction of the liver with systemic T cells

In the remainder of this review, I consider the interactions between the liver and conventional $CD4^+$ and $CD8^+$, αβ T cells. Circulating T cells pass through the liver sinusoids and can interact with Kupffer cells and LSECs. Some experiments indicate further that naive T cells might interact with hepatocytes. Antigens that are expressed in the liver might be taken up by immature DCs, and might then be presented to $CD4^+$ and $CD8^+$ T cells, either in lymphoid-tissue aggregates in the portal tracts or in secondary lymphoid tissues. Alternatively, antigens might be recognized *in situ* on LSECs, Kupffer cells and, possibly, hepatocytes. The outcome of antigen recognition in the liver could be full T-cell activation, immune deviation leading to the differentiation of T cells to a suppressive or regulatory phenotype, or abortive activation leading to T-cell apoptosis. In addition, the liver might sequester activated T cells in an antigen-independent manner, and the high apoptotic rate of such cells has given rise to the idea that the liver might be a 'graveyard' for systemic T cells[72]. Inflammation of the liver due to viral hepatitis is accompanied by the upregulation of expression of an extensive panel of T-cell interaction molecules, including intercellular adhesion molecule 1 (ICAM1), MHC class II molecules[73], vascular cell adhesion molecule 1 (VCAM1)[74], co-stimulatory molecules of the B7 family[75] and CD95 (FAS)[76]. These molecules might modify cell trafficking, priming and the induction of tolerance.

Cell trafficking

In most blood vessels, leukocytes flow past the endothelium without forming adhesions. Under inflammatory conditions, selectins expressed by leukocytes interact with addressins expressed by the endothelium to initiate 'rolling', which slows the cells and allows other adhesion molecules to become engaged[77]. *In vivo* microscopy shows that the blood flow in hepatic sinusoids is slow and intermittent, facilitating interactions between blood cells and the endothelium, which might, therefore, be independent of selectin–addressin interactions[78]. LSECs express several adhesion molecules, including a high density of ICAM1 and ICAM2 (REF. 79), and vascular adhesion protein 1 (VAP1)[80]. The reason for this constitutive expression of adhesion molecules is unknown, but one factor might be that LPS from the intestine interacts with Toll-like receptor 4 (TLR4), which is expressed in the liver[81], probably by LSECs[82]. This interaction between LPS and liver cells increases the level of expression of adhesion molecules[83]. The ICAMs engage lymphocyte function-associated antigen 1 (LFA1) on activated T cells, whereas VAP1 binds to unknown ligand(s) on $CD8^+$ T cells and on $CD16^+$ cells, including NK cells. Therefore, the adhesion molecules that are expressed by LSECs give the liver a dual T-cell tropism: for activated T cells in preference to resting T cells, and for $CD8^+$ T cells and NK cells in preference to $CD4^+$ T cells.

In addition to lymphocytes, DCs also traffic through the liver, passing from blood in the sinusoids to the space of Disse, from there through lymphatics in the portal tracts, and ultimately to the hepatic and celiac lymph nodes[84]. The migration of DCs seems to be controlled by chemokines[85,86] (FIG. 4). During experimental infection with *P. acnes*, blood-bourne DC precursors expressing CC-chemokine receptor 1 (CCR1) and CCR5 formed intrahepatic granulomas in response to CCL3. After maturation, the DCs expressed CCR7 and became responsive to CCL21 (secondary lymphoid tissue chemokine, SLC), which promoted their migration to organized lymphoid tissue[87].

Antigen presentation

The liver can present antigen to T cells through several mechanisms. The Kupffer cells form an antigen-trapping system, and the liver is patrolled by 'professional' APCs in the form of immature DCs. In humans, $CD45^+$ cells with high-level expression of MHC class II molecules and a dendritic morphology can be seen in the portal tracts, but these cells are unusual in that they lack expression of CD11C, which is usually found on DCs[88]. In mice, two populations of DC exist, which are distinguished by their expression of CD8α. The DCs that do not express CD8 have been termed 'myeloid' DCs, whereas DCs that express CD8α have been termed 'lymphoid' DCs. However, it is not clear that these cell types originate from distinct lineages[89]. Both types of DC are present in the mouse liver, and both can differentiate into effective APCs *ex vivo*[90]. Trafficking immature mouse DCs pass through the liver, and while they are in the hepatic sinusoids, these DCs are likely to interact with Kupffer cells[91], in part through their expression of lectin-like carbohydrate receptors[92,93]. This interaction might be facilitated by CCL3 synthesized by the Kupffer cells, which communicates with the DCs through their CCR1 chemokine receptors (FIG. 4).

Box 2 | **Experimental models of immune-mediated liver damage**

Injection of the lectin concanavalin A (ConA) into mice causes T-cell-dependent liver damage. This model depends on $CD4^+$ T cells, interferon-γ and natural killer cells. The difficulty in interpreting this model results from the fact that ConA binds many ligands and might have unknown effects in the liver in addition to recruiting and activating T cells[165,166].

The infusion of non-tolerant T cells into a transgenic mouse that expresses an MHC class I molecule on hepatocytes results in transient hepatitis, accompanied by apoptosis of the T cells[103,167]. The difficulty in interpreting this model lies in the difficulty of knowing on which cells the MHC transgene is expressed.

Transgenesis has been used also to express the entire genome of hepatitis B virus (HBV) and of hepatitis C virus in hepatocytes. Such mice develop tolerance to virus-encoded proteins, but the infusion of non-tolerant T cells causes liver inflammation[168,169]. Interpretation of this model is complex, because although the viral antigens are targeted to hepatocytes, cross-presentation might occur on liver sinusoidal endothelial cells, Kupffer cells or dendritic cells. Virus proteins might have evolved to cause immune effects and/or to have direct cytopathic effects, which makes models of this kind both realistic and difficult to unravel. In the HBV-transgenic model, the suppression of virus RNA was due to cytokines, rather than cytotoxic mechanisms.

A simple model of 'pure' T-cell-mediated liver damage has been developed by the injection of specific antigenic peptides into T-cell-receptor-transgenic mice[142,143]. In this model, there are no restrictions on the site of antigen presentation, which makes models that are based on the roles of different antigen-presenting cells difficult to test. Crossing the transgenic mice to a background that lacks death receptor(s) has shown their importance for this kind of liver damage.

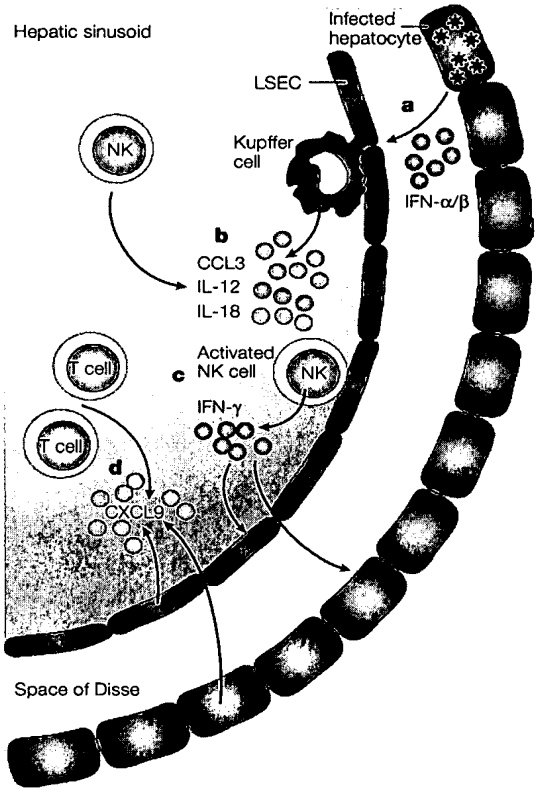

Figure 3 | **The cytokine/chemokine cascade through which NK cells recruit T cells.** The steps are: **a** | many cell types, including hepatocytes, synthesize type 1 interferons (IFN-α/β) in response to virus infection; **b** | Kupffer cells respond to IFN-α and IFN-β by producing CCL3, which recruits natural killer (NK) cells; **c** | NK cells, once activated by the Kupffer-cell products interleukin-12 (IL-12) and, perhaps, IL-18, produce IFN-γ, which causes tissue cells, including hepatocytes and liver sinusoidal endothelial cells (LSECs), to produce CXCL9; **d** | CXCL9 recruits T cells.

An interaction between DCs and macrophages might be important for CROSS-PRESENTATION — for example, of antigen derived from the phagocytosis of apoptotic Kupffer cells[94,95] — and also for priming of the immune response to pathogens.

The *in vivo* function of DCs might lead to effective T-cell priming or to T-cell tolerance. The evidence for tolerance induction by some DCs is clear, and one important distinction between DCs that induce immunity and those that induce tolerance is that the former secrete IL-12, whereas the latter secrete IL-10. As I discuss later, secretion of IL-10 is prevalent in normal liver. Another important issue is whether the DCs that are located in the portal tracts are involved actively in antigen presentation at that site or whether they must traffic to lymph nodes first.

In addition to DCs and Kupffer cells, antigens passing through the hepatic sinusoids encounter LSECs, which form a highly distinctive endothelium. Mouse LSECs express molecules that promote antigen uptake, including the mannose receptor and the scavenger receptor, and molecules that promote antigen presentation, including CD40, CD80 and CD86 (REF. 96). These cells are, therefore, equipped to act as APCs, and they present antigens to both $CD4^+$ and $CD8^+$ T cells (FIG. 5). Despite their expression of co-stimulatory molecules, the most common consequence of T-cell priming by LSECs is tolerance[97,98]. The reason for this paradoxical behaviour is unknown. By contrast, human LSECs express CD40, but they do not seem to express CD80 or CD86 constitutively. These molecules are, however, expressed during inflammation[99]. So, the APC properties of mouse and human LSECs might differ.

The sinusoidal endothelium is fenestrated. Small holes, clustered together to form sieve plates, facilitate the diffusion of metabolites backwards and forwards between the blood and hepatocytes, and scanning electron micrographs have indicated that there are also gaps between the LSECs. This creates the possibility that both naive and previously activated T cells could gain direct access to hepatocytes. This would be a unique situation, because resting naive T cells do not have access to most tissues, which are selectively populated with long-lived memory T cells[100,101]. The extent to which such tissue access occurs in the liver is controversial. The selective accumulation of antigen-specific T cells in the livers of transgenic mice expressing either the HBV genome[102] or a non-self MHC class I molecule[103] is compatible with the idea that such direct access to hepatocytes occurs in the living liver. However, these data are open to alternative interpretations. The HBV antigens could have been subject to cross-presentation, for example by LSECs, whereas the non-self MHC class I molecules could have been ectopically expressed.

T-cell tolerance due to apoptosis

The infusion of non-tolerant $CD8^+$ T cells into transgenic mice that express a non-self MHC class I alloantigen on hepatocytes results in the rapid and selective accumulation of these T cells in the liver. The T cells become activated and might undergo several cell divisions, but ultimately, they die by apoptosis[104,105] (FIG. 5b). In mice that contain antigen-specific TCR-transgenic $CD8^+$ T cells and are injected systemically with soluble antigenic peptide, activation and proliferation of the T cells in lymphoid tissues is followed by the accumulation and apoptosis of these T cells in the liver[25,30]. Finally, transplantation of an allogeneic liver into both humans and mice results in T-cell infiltration, followed by apoptosis of the infiltrating cells[106,107]. To investigate the basis of these observations, naive T cells have been cultured with purified alloantigenic hepatocytes, which results in partial activation of the T cells, followed by their apoptosis. This is believed to be an example of a type of apoptosis known as PASSIVE CELL DEATH (PCD).

In mature T cells, PCD can occur when fully activated T cells are deprived of antigen, or of growth and survival factors such as IL-2. This seems to be a main mechanism for the clearance of expanded clones of T cells after antigen has been cleared during and after an acute infection, whereas FAS-induced apoptosis is less important in this situation[108]. The mechanism of PCD involves loss of mitochondrial membrane integrity,

CROSS-PRESENTATION
The process by which exogenous antigens that are expressed by one cell are processed and presented by MHC class I molecules of another cell. Peptides derived from antigenic proteins are susceptible to this form of presentation, whereas MHC alloantigens are not. Dendritic cells and liver sinusoidal endothelial cells are particularly efficient at cross-presentation.

PASSIVE CELL DEATH
The death of T cells due to activation in the absence of sufficient survival signals, or when antigen is cleared and signals through the T-cell receptor cease.

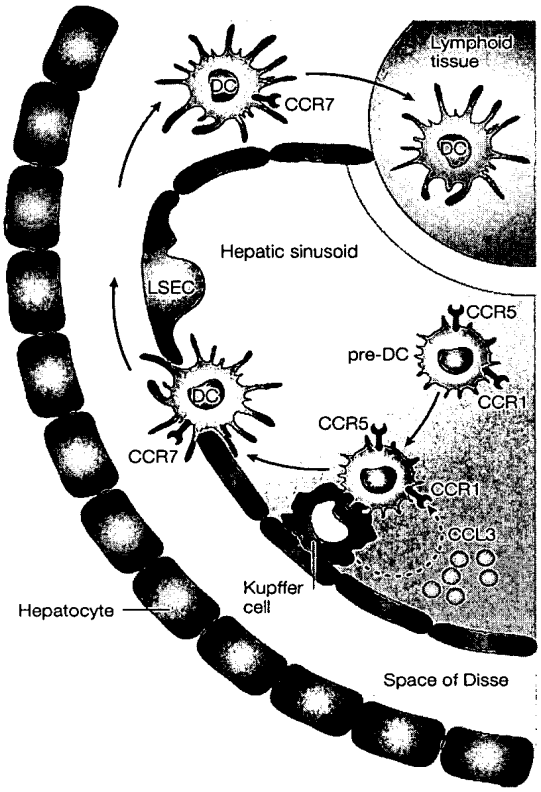

Figure 4 | **Trafficking and interactions of dendritic cells in the liver, controlled by chemokines.** In response to inflammatory signals, Kupffer cells secrete CCL3, which engages CC-chemokine receptor 1 (CCR1) on immature dendritic cells (pre-DCs). Maturation of these cells results in the loss of expression of CCR1 and CCR5, and the expression of CCR7. The DCs become responsive to CCL21, which is produced by lymphatic endothelia and tissue stroma, both in lymph nodes and in the portal tracts. DCs leave the hepatic sinusoids and migrate to lymphoid aggregates in the portal tracts or to lymph nodes. LSEC, liver sinusoidal endothelial cell.

the release of cytochrome *c* and the activation of pro-caspase-9 through apoptotic protease-activating factor 1 (APAF1)[109,110]. Once activated, caspase-9 cleaves and activates pro-caspase-3 and -7, which yields the effector caspases that are responsible for the breakdown of many crucial substrates, including other caspases[111]. In contrast to PCD, ACTIVATION-INDUCED CELL DEATH (AICD) occurs in fully activated T cells and is promoted by IL-2 (REFS 112,113). This process depends on the expression of death receptors, such as FAS and tumour-necrosis factor receptor 1 (TNFR1), and on activation of the death-receptor-initiated caspase cascade, which occurs in part by the downregulation of expression of the inhibitory molecule FLIP (FLICE-like inhibitory protein)[114]. After their ligation, death receptors assemble with adaptor proteins to form a death-inducing signalling complex (DISC), the effect of which is to cleave and activate pro-caspase-8 (REF. 115). Activated caspase-8 cleaves pro-caspase-3, leading to convergence of the PCD and AICD pathways[111].

ACTIVATION-INDUCED CELL DEATH (AICD). The apoptosis of fully activated T cells, mediated by ligation of death receptors — such as CD95 (FAS), tumour-necrosis factor receptor 1 (TNFR1) and TNF-related apoptosis-inducing ligand receptor (TRAILR) — on their surface.

Experiments in which hepatocytes induce the abortive activation, followed by death, of naive T cells might be examples of PCD. The addition of exogenous IL-2 inhibits the death of T cells that are co-cultured with hepatocytes, which supports the theory that this form of cell death is PCD[105]. Mouse hepatocytes, unlike mouse LSECs, do not express CD80 and CD86 (REF. 116), which indicates that PCD might be induced by the presentation of antigen in the absence of normal co-stimulatory ligands — the same conditions that would cause anergy of a previously activated T-cell clone[117]. A role for PCD due to lack of co-stimulation in liver allograft tolerance *in vivo* was indicated by experiments in which liver-grafted mice were treated with cytotoxic T-lymphocyte antigen 4 (CTLA4)–immunoglobulin, which is an inhibitor of CD80/CD86-mediated co-stimulation. In the CTLA4–immunoglobulin-treated mice, enhanced graft survival was associated with the increased apoptosis of graft-infiltrating $CD4^+$ and $CD8^+$ T cells[118].

If T cells die in the liver by PCD, death receptors such as FAS and TNFR1 would be expected to be irrelevant. One clear example contradicts this and provides evidence in favour of an AICD model. The $CD8^+$ T-cell infiltrate in livers infected with a recombinant adenovirus vector was cleared by a Fas-dependent mechanism[119]. This result argues against a purely passive interpretation of T-cell death in the liver. Such AICD-based models might depend on systemic T-cell activation, because such activation induced by super-antigens results in the upregulation of expression of Fas ligand (FasL) in several tissues, including the liver[120].

How can the liver induce both PCD and AICD of $CD8^+$ T cells? The answer might be that AICD depends on full T-cell activation and differentiation. In most of the models that I discuss, T-cell activation is not optimum. Examples include the transgenic expression of non-self MHC molecules on hepatocytes and a liver transplant in the absence of infection. Although the transplant procedure might result in tissue damage and some inflammation, the antigen-presenting system must not be sufficiently perturbed to change to an immunogenic state. Both of these situations, therefore, seem to result in PCD of $CD8^+$ T cells. By contrast, in the adenovirus model, there was evidence for the involvement of Fas and, therefore, of AICD. So, it is probable that the liver kills $CD8^+$ T cells by two mechanisms. Direct recognition of antigen on LSECs, and possibly also on hepatocytes, by naive $CD8^+$ T cells results in partial activation of the T cells, followed by PCD (FIG. 5b). By contrast, recognition of liver antigens by $CD8^+$ T cells in the context of an adenovirus infection results in full T-cell activation, rendering the T cells susceptible to AICD. The role of AICD in the disappearance of $CD8^+$ T-cell infiltrates in other models of viral infection is unexplored, although an unusual variation of AICD might occur in liver $CD8^+$ T cells that are specific for *Listeria monocytogenes*, the clearance of which depends on perforin[121].

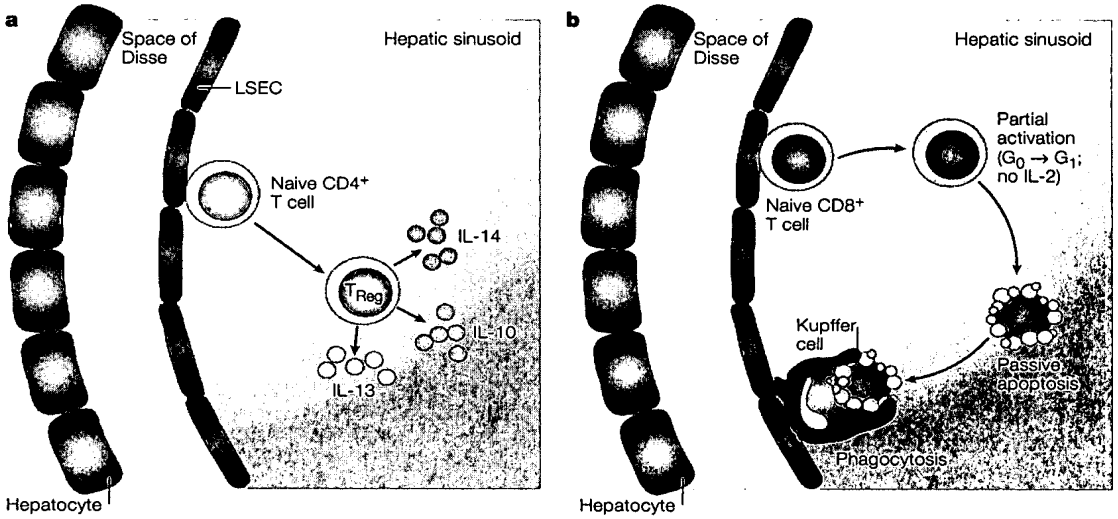

Figure 5 | **Induction of T-cell tolerance by interaction of T cells with liver sinusoidal endothelial cells. a** | Interaction of naive CD4+ T cells with liver sinusoidal endothelial cells (LSECs) results in differentiation of the T cells to a regulatory (T_{Reg}) phenotype. This is probably because LSECs normally produce interleukin-10 (IL-10), which favours the regulatory pathway of CD4+ T-cell differentiation. **b** | Interaction of naive CD8+ T cells with sinusoidal endothelium results in partial activation of the T cells, followed by passive cell death. The reason for this is unknown, but I propose that continuous exposure to trace amounts of intestinal lipopolysaccharide (LPS) results in the differentiation of both LSECs and liver DCs to a state that promotes different forms of T-cell tolerance in CD4+ and CD8+ T cells. The apoptotic CD8+ T cells are then endocytosed by Kupffer cells.

Resting memory T cells seem to be resistant to the pro-apoptotic effects of the liver. So, virus-specific memory CD8+ T cells are found in this site, as are TCR-transgenic memory CD4+ T cells specific for a protein antigen[100,101]. For these cells, the liver might not be a killing field, but rather a hospitable neutral zone.

Immune suppression, regulation and deviation

These two mechanisms to promote CD8+ T-cell apoptosis are not the only tolerogenic processes that are active in the liver. Soluble antigens passing through the sinusoids are taken up by LSECs and presented to both CD8+ T cells and CD4+ T cells. T cells that are primed by LSECs might become activated and undergo proliferation, but they fail to sustain secretion of IL-2 and IFN-γ. CD8+ T cells that are primed by LSECs fail to differentiate into cytotoxic effector cells, whereas CD4+ T cells that are primed by LSECs might differentiate towards an anti-inflammatory (IL-4- and IL-10-secreting) phenotype[97] (FIG. 5a). The local synthesis of IL-10 by T cells primed by LSECs might be important, because IL-10 alters the expression of chemokine receptors by DCs in ways that would be expected to disrupt their homing to lymphoid tissues. Specifically, IL-10 causes the increased expression of CCR5 and the decreased expression of CCR7 by DCs, which renders them less responsive to the lymphoid-tissue chemokine CCL21 (REFS 122,123). The synthesis of IL-10 is prevalent in the liver. In addition to LSECs, mouse liver-derived DCs also preferentially induce the synthesis of IL-10 by CD4+ T cells, in contrast to bone-marrow-derived DCs, which preferentially induce IFN-γ synthesis[90]. In rat liver allografts, an early phase of T-cell apoptosis is followed by the accumulation of $CD4^+CD45RC^{low}$ cells that produce IL-13 (REF. 124). So, although liver allografts seem to be protected in the acute phase by apoptosis of host CD8+ T cells, they might survive in the long term due to the presence of host CD4+ regulatory T cells.

What overcomes base-line tolerance?

I have described the liver as an organ in which the antigen-presenting capacity of LSECs and many DCs is biased strongly towards the induction of CD4+ T cells with a regulatory phenotype, whereas both CD8+ T cells that are activated systemically that localize to the liver, and naive CD8+ T cells that first encounter antigen in the liver, are predisposed strongly to undergo apoptosis. The problem is to understand how this default state of tolerance might be reversed to allow priming of T cells to occur and effector responses to be delivered. The solution is not self-evident, but there are some clues.

Type 1 interferons (IFN-α/β) activate a cytokine/chemokine cascade that recruits T cells during virus infections[34,35]. Such IFN synthesis occurs in tissue cells, including hepatocytes, in response to virus infection. In addition, type 1 IFNs induce the synthesis of IL-15, which promotes the survival of CD8+ T cells[125,126]. So, synthesis of type 1 IFNs might be an important event that overcomes liver tolerance and allows an intrahepatic T-cell response to proceed.

A second factor might be the regulation of DC function. The maturation of DC precursors seems to be a two-stage process. Initial maturation results in upregulation of the antigen-processing machinery (including MHC class I and II molecules), expression of co-stimulatory molecules (CD80 and CD86) and the synthesis of IL-10. DCs at this developmental stage prime regulatory T cells preferentially, and their

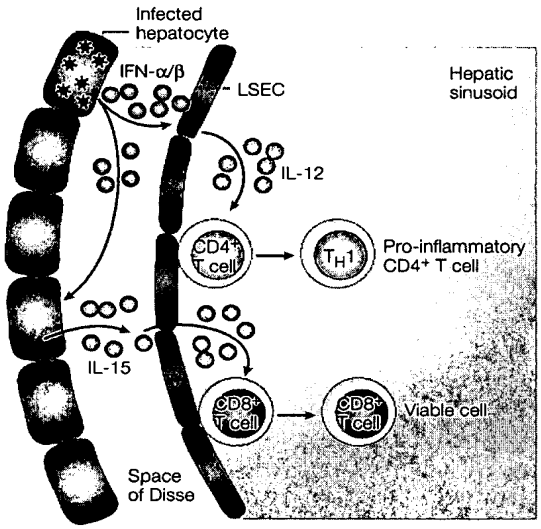

Figure 6 | **An hypothesis to explain how the tolerant state of the liver is reversed, resulting in T-cell priming and immunity.** This hypothesis gives type 1 interferons (IFN-α/β) a central role in the normal liver, and it proposes that chronic exposure to lipopolysaccharide results in the desensitization of sinusoidal endothelial cells to activation signals. These cells present antigen in the context of co-stimulatory molecules, but do not secrete pro-inflammatory cytokines. Therefore, $CD4^+$ T cells become regulatory, whereas $CD8^+$ T cells undergo passive cell death, as shown in FIG. 5. As shown here, under the influence of type 1 IFNs in an infected liver, everything changes. A virus-infected hepatocyte secretes type 1 IFNs, which act on liver sinusoidal endothelial cells (LSECs), causing them to secrete interleukin-12 (IL-12) and promote the differentiation of $CD4^+$ T cells to inflammatory T helper 1 (T_H1) cells. In parallel, type 1 IFNs act on other parenchymal cells, causing them to secrete IL-15, a survival factor for $CD8^+$ T cells. Not shown in this figure is the type 1 IFN-initiated cytokine/chemokine cascade that promotes T-cell recruitment (FIG. 3).

phenotype and functions are markedly similar to those of LSECs. By contrast, the final maturation of DCs results in the synthesis of IL-6, IL-12 and tumour-necrosis factor (TNF), and such DCs prime $CD4^+$ T cells to deliver an immune response[127]. The first maturation step is induced by diverse signals, including exposure to elements of the gut flora[128], whereas the second step is induced by exposure to LPS[129]. I have already considered the effects of intestinal LPS on promoting the expression of adhesion molecules in the liver[83], and this effect indicates that LPS should also promote the final maturation of liver DCs. However, liver DCs prime $CD4^+$ T cells to have a regulatory function[90]. How can this be explained?

I would like to put forward the argument that tolerance-inducing liver DCs are in a state of partial LPS resistance, brought about by continuous exposure to LPS. So, they are refractory to final maturation signals through TLR4 and remain in a state that promotes the differentiation of regulatory T cells. If this is the case, a distinct signal must be required to promote final DC maturation and, thereby, the priming of T cells to deliver effector function. This requirement is met by type 1 IFNs. In addition to its other functions, which have been discussed already, IFN-α promotes DC maturation[130,131], and the type 1 IFNs have been proposed to be a crucial link between innate and adaptive immunity[132].

On the basis of this discussion, I propose the following model. First, the main reason for $CD4^+$ T-cell tolerance induced by antigen presentation in the liver is that both DCs and LSECs respond to the continuous presence of LPS of intestinal origin by becoming unresponsive to TLR4 signals and by assuming a differentiation state that promotes the activation of $CD4^+$ regulatory T cells. This has been shown for LSECs by Knolle *et al.*[133], and I propose that it applies to liver DCs also. Second, the main reason for $CD8^+$ T-cell tolerance in the liver is that liver DCs and LSECs cause partial activation of $CD8^+$ T cells, leading to PCD. Data from my own laboratory show an important role for LSECs in inducing $CD8^+$ T-cell trapping and death[25]. Third, pathogens that elicit a type 1 IFN response promote naive T-cell recruitment, switch the function of liver DCs and LSECs so that they promote the differentiation of $CD4^+$ T cells, and allow full activation and survival of $CD8^+$ T cells (FIG. 6). Thereby, an effective immune response will be delivered. Finally, it follows that pathogens that persist in the liver either suppress type 1 IFN production or evade the effects of type 1 IFNs.

FAS expression and hepatocyte damage

The location of the liver downstream from the huge absorptive surface of the gastrointestinal tract places it in the front line in relation to toxic chemicals, and the liver has evolved the capacity to detoxify potentially dangerous molecules, often through conjugation to glucuronic acid followed by excretion into the bile. However, the uptake of such molecules by hepatocytes poses a constant risk of DNA damage, which could lead to mutation and carcinogenesis. The capacity to induce rapid apoptosis of mutant hepatocytes might explain why hepatocytes express FAS on their membranes. In mice, the Fas signalling pathway is active in these cells, and they are always poised to undergo apoptosis; this can be shown by the *in vivo* injection of Fas-specific antibodies[134] or by the co-culture of hepatocytes with FasL-expressing cells[135]. This capacity to undergo apoptosis is linked to the potential for extensive regeneration, which is manifested as recovery after acute hepatitis, liver regeneration after partial hepatectomy[136] and the restoration of full hepatocyte mass after the transplantation of a 'split liver'[137]. The regeneration of the liver is linked closely to its resident lymphocytes. During the early stages of liver regeneration, there is a rapid, transient increase in the proportion of resident NKT cells[66]. This raises the possibility that, similar to other populations of T cells with a restricted TCR repertoire[138,139], these cells might be involved in the response to tissue damage. However, the role of lymphocytes in liver regeneration is unclear, and NK cells have been proposed to both support[140] and suppress[141] regeneration.

The expression of Fas by mouse hepatocytes places them at risk of acute damage whenever activated lymphocytes accumulate in the liver. In the simplified model of systemic $CD8^+$ T-cell activation induced by the injection of antigenic peptide into TCR-transgenic mice, T-cell accumulation and apoptosis in the liver was accompanied by hepatocyte damage, resulting in histological lesions and elevation of the level of serum aminotransaminases[30,142]. Such damage was reduced in Fas-deficient mice[143], which indicates that FasL — either on the $CD8^+$ T cells themselves or induced by their presence — engages Fas on the hepatocytes. In humans, the situation is more complicated. The basal level of expression of FAS by human hepatocytes is lower than in mice, but it is elevated in inflammatory conditions, including infection with HBV or HCV[144]. This does not seem to be associated with hepatocyte death. In fact, the expression of FAS in the infected liver might be part of antiviral defences during infection with HCV, because it is negatively correlated with liver damage, as assessed by the level of alanine aminotransaminase[145]. FAS expression is also a good predictor of the response to IFN-α therapy[146]. *In vivo*, the limiting factor might be the local expression of FASL. T cells could potentially provide FASL in two ways: by its expression on activated T cells[147,148] or by an immune interaction with hepatocytes in which the ligation of CD40 induces the expression of FASL by the hepatocytes themselves[149]. This might be what is happening when mice are treated with a superantigen, which induces the expression of FasL on many tissues, including the liver[120].

Concluding remarks

The global prevalence of infections of the liver, including malaria, HBV and HCV, makes it important that we understand the distinctive constraints on T-cell immunity in this organ. Vaccines against malaria and HCV are still elusive, and the large number of people who are infected with liver pathogens makes therapy, as well as prevention, an important goal of immunological intervention. Antigen presentation by LSECs, and possibly by hepatocytes, might frustrate efforts to prime an effective immune response, and pro-apoptotic mechanisms might eliminate activated liver-specific $CD8^+$ T cells. Highly activated T cells are dangerous in the liver, because hepatocytes express the FAS death receptor and are susceptible to immunopathology. In the face of these obstacles, it is highly desirable that we devise strategies to modify the natural history of such chronic infections by manipulating the immune system to deliver protective responses without unacceptable immunopathology.

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Online links

DATABASES

The following terms in this article are linked online to:

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hepatitis A virus | hepatitis B virus | hepatitis C virus | *Listeria monocytogenes* | mouse hepatitis virus | *Mycobacterium tuberculosis*

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APAF1 | B220 | caspase-8 | caspase-9 | CCL3 | CCL21 | CCR1 | CCR5 | CCR7 | CD1d | CD2 | CD7 | CD8α | CD11C | CD25 | CD40 | CD56 | CD69 | CD80 | CD86 | CD95 | CTLA4 | CXCL9 | FLIP | ICAM1 | ICAM2 | IFN-α/β | IFN-γ | IL-2 | IL-2Rβ | IL-4 | IL-6 | IL-10 | IL-12 | IL-15 | LFA1 | NK1.1 | RAG1 | RAG2 | TLR4 | TNF | TNFR1 | VAP1 | VCAM1

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Fetal gene transfer

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Gene transfer early in development for the treatment of monogenetic and other diseases could overcome major obstacles of intervention in the mature individual. Early gene transfer may prevent the onset of irreversible pathological changes, predispose the individual to immunological tolerance to the introduced protein, take advantage of the high vector to cell ratio, and provide unique access to stem cell/progenitor compartments. The past few years have witnessed the publication of five studies showing long-term correction of monogenetic disorders by fetal gene transfer. Many others have examined the use of new vector systems with therapeutic transgenes, tested their potential for treating diseases in a wide range of organs (including the brain, lung and skin), and examined the hazards of fetal application. This review gives a comprehensive summary of the development of fetal gene transfer over the past few years.

Keywords Adeno-associated virus, adenovirus, fetal gene therapy, in utero gene transfer, lentivirus

Introduction

In 1984, Jaenisch and coworkers explored the potential use of retroviral vectors to deliver transgenes to fetal animal tissue. They injected mid-gestation mouse embryos with a cell line engineered to produce retrovirus carrying a marker gene [1]. Expression of the marker gene was detected in various tissues of adult mice, albeit in low amounts. However, these experiments were not conducted with a therapeutic goal and it took another five years for researchers at the NIH to successfully performed fetal gene therapy. After transducing fetal sheep hematopoietic cells *ex vivo* with a retroviral vector carrying a marker gene and re-infusing these cells into the fetus, transgene expression was detected for over a four-year period [2,3]. The concept and advantages of fetal gene therapy were highlighted again in 1995 by several studies on *in vivo* gene delivery in fetal animals [4].

In 1998, the first pre-protocols for human prenatal gene therapy were submitted to the Recombinant DNA advisory Committee. The choice of the two candidate diseases, α-thalassemia and severe combined immunodeficiency caused by adenosine deaminase deficiency (ADA-SCID) were convincingly justified [5]. Four advantages of fetal versus adult gene therapy were hypothesized in this pre-proposal: (i) assuming that stem cells are the principal target, their rapid expansion *in utero* makes them accessible to retroviral vectors and provides a large pool of genetically altered cells; (ii) the small size of the fetus is advantageous when scaling up from therapy in small animals to large animals or humans; (iii) the immunological immaturity of the fetus may result in a reduction in, or even absence of immune/inflammatory reactions against vector and transgenic protein and may permit acquisition of tolerance to the latter; and (iv) the tissue barriers, including the endothelium and epithelium, may be more readily penetrated by vectors in the fetus than in the adult, allowing more extensive vector transduction of organs such as the brain and skin. Therefore, fetal gene therapy could achieve permanent disease correction where adult gene therapy might fail [5].

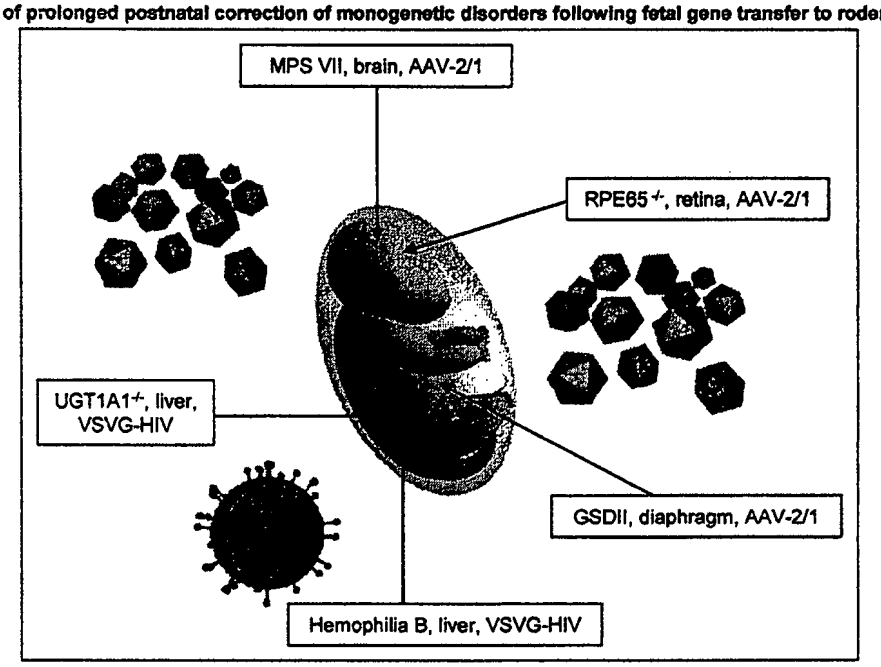
Preclinical studies

Proof-of-concept

Early attempts to achieve therapeutic gene transfer were reported in 1997 and 1999. One report, describing reversal of lethality in a mouse model of cystic fibrosis after intra-amniotic injection of adenovirus carrying cystic fibrosis transmembrane conductance regulator (CFTR) cDNA, was met with substantial criticism and was not repeatable by other researchers [6]. Another report described transient Factor VIII expression (< 21 days) in haemophilia A mice after injection of adenovirus [7].

Over the past four years, five studies in rodent models have emerged to show proof-of-concept for a postnatal therapeutic effect of *in utero* gene delivery. These are depicted in Figure 1 showing the route of injection, the candidate disease, and the vector implemented. Rucker *et al* used an adeno-associated virus (AAV) serotype 1 vector (AAV-2/1) to deliver human α-glucosidase to the diaphragm of mice deficient in this enzyme, a condition which often results in death from respiratory failure in humans [8••]. Normal contractile function was restored for up to 6 months postpartum. In 2005, Dejneka *et al* used the same AAV serotype to deliver human retinal pigment epithelium 65 (RPE65) to the retinal pigmented epithelium of Rpe65-/- mice and demonstrated restoration of visual

Figure 1. Studies of prolonged postnatal correction of monogenetic disorders following fetal gene transfer to rodents.



Adeno-associated virus (**AAV**) vectors have been used for the treatment of models of mucopolysaccharidosis VII (**MPS VII**) [10••], Leber congenital amaurosis [9••] and glycogen storage disease type II (**GSDII**; Pompe disease) [8••]. HIV-lentivirus vectors have been used to correct models of Factor IX deficiency (hemophilia B) [11••] and bilirubin UDP-glucuronyltransferase deficiency (**UGT1A1**; Crigler-Najjar type I) [12••]. **RPE65** retinal pigment epithelium 65, **VSVG** vesicular stomatitis virus G.

function [9••]. Karolewski and Wolfe also used AAV-2/1 to treat a mouse model of mucopolysaccharidosis type VII. This vector carrying the human β-glucuronidase cDNA was injected into the ventricle of the fetal brain and resulted in widespread gene expression in the brain and spinal cord and a significant improvement in survival after one year [10••].

All three studies used non-integrating vectors for gene delivery to tissues which undergo minimal cell cycling in adult life. In contrast integration-proficient HIV vectors pseudotyped with vesicular stomatitis virus G (VSVG) were used to correct models of Factor IX deficiency (hemophilia B) in mice, bilirubin uridine diphosphate-(UDP) glucuronyltransferase (UGT1A1) deficiency (Crigler-Najjar type I) in Gunn rats, and Leber congenital amaurosis in chickens. In the first study, Waddington *et al* injected the vector into the fetal vitelline vessels, which resulted in predominant expression of human Factor IX in the mouse liver (the site of endogenous synthesis) and achieved lifelong correction of the bleeding diathesis [11••]. In the second study, Seppen *et al* achieved intraperitoneal and hepatic delivery of the transgene from direct intrahepatic vector injection. This significantly ameliorated the metabolic defect for at least one year [12••]. In both of the above studies, long-term detection of clusters of transduced cells in liver and intestine, respectively, indicated that organ-specific progenitors had been transduced and were a source of transgene-expressing cells over the rodent's lifetime. In a third study, Williams *et al* describes long-term correction of a chicken model of Leber congenital amaurosis by lentiviral gene transfer of retinal guanylate cyclase-1 to the neural tube of the embryonic chick [13•]. Early intervention was necessary to pre-empt and prevent photoreceptor cell dysregulation, which precedes degeneration of these cells. These reports of long-term therapeutic benefit of *in utero* gene transfer are supported by several exciting investigations toward fetal gene therapy, which are detailed below.

Consistent with studies demonstrating induction of tolerance to transgenic proteins expressed after fetal gene [14] or stem cell [15] transfer, Sabatino *et al* have demonstrated tolerization to human Factor IX after *in utero* or neonatal administration of AAV [16]. This is supported by Toelen *et al*, who showed that following delivery of a luciferase transgene to rats by various routes of HIV-lentivirus injection, long-term expression of more than 30 weeks was observed [17].

In a model of congenital diaphragmatic hernia induced by nitrofen in rats, Larson and Cohen reported that following delivery of human CFTR to the fetal lungs by intra-amniotic injection of an adenovirus vector, pulmonary hypoplasia (as measured by several parameters of lung pathology including lung volume, internal lung surface area, and number of lung saccules) was ameliorated [18•]. This non-integrating vector was eliminated by dilution from high cell turnover in the respiratory epithelium, after providing the requisite transient expression.

Han *et al* recently reported a study in which therapeutic correction was attempted in an α-thalassemia mouse model by administration of lentivirus at two-thirds gestation [19•]. Although in some animals, expression of α-globin reached up to 20%, this was lost after half a year. This may be symptomatic of a failure to transduce enough erythroid progenitors and/or evidence of transcriptional silencing.

Bilbao *et al* compared AAV-2/1 and AAV-2 after intraperitoneal, intramuscular and intravascular administration *in utero* and established that AAV-2/1 produced the highest transgene expression in the muscle [20]. First-generation and helper-dependent (high capacity/gutless) adenoviral vectors were also compared after intramuscular administration. Increased persistence of vector genomes and reduced toxicity were noted after administration of the helper-dependent adenovirus. These researchers proceeded to show that this vector could be used to deliver full-length murine dystrophin cDNA to fetal *mdx* mice (a mouse model of Duchenne muscular dystrophy). Nine weeks post-delivery, partial restoration of the dystrophin glycoprotein complex in the hind limbs of these mice was demonstrated [21]. Muhle *et al* applied a combination of adenovirus and AAV-2 to a mouse model of Herlitz junctional epidermolysis bullosa [22•]. Both vectors, carrying the cDNA for β3 chain of laminin-5 protein, were administered into the amniotic fluid. Although laminin-5 was detectable in the basement membranes of skin and mucosae, only a minor increase in survival was observed. Bowers *et al* developed a novel vector combining the high payload capacity of HSV-derived amplicon with the ability of the Sleeping Beauty transposon to mediate integration into the host genome [23]. *In utero* intraventricular injection of this vector resulted in extensive marker gene expression in neuronal precursors for more than 3 months.

Koyama *et al*, Kimura *et al*; and Nakamura *et al* have presented an innovative approach to the gene therapy of pregnancy-related conditions [24-27]. This is based on gene delivery to the extra-embryonic membranes, placenta and uterus, which at least in the first two tissues constitutes bona fide gene transfer to fetal rather than adult tissue. DNA was complexed with Sendai virus hemagglutinating virus of Japan (HVJ) envelope and injected into the uterine cavity but outside the amniotic sac. Expression was observed transiently (for 3 days) in various tissues, including the uterus, placenta, decidual membrane and fetal membrane [24-26]. Transient overexpression of human VEGF was achieved which temporarily reduced the systemic blood pressure of the dam. The researchers suggested that this might be a strategy for treating pre-eclampsia [27].

Compared with *in vivo* vector application, research on *ex vivo* transduction of stem cells before implantation has progressed more slowly and is reviewed more fully in reference [28]. Since this review was published in 2004, Rio *et al* have demonstrated significant engraftment in mice following *in utero* transplantation of hematopoietic stem cells from congenic donors after *ex vivo* transduction with a retroviral vector encoding green fluorescent protein (GFP) [29]. Marker gene expression was detectable in the peripheral blood cells of these animals as well as in those of secondary recipients transplanted with bone marrow from the *in utero* tranplanted mice. Chan *et al* demonstrated engraftment of lentivirus-transduced human fetal mesenchymal stem cells for at least 4 months after injection into *mdx* mice *in utero* [30]. This is particularly interesting, and may be related to the specific immunosuppressive property of mesenchymal stem cells, as Peranteau *et al* have recently demonstrated an immune barrier to allogeneic but not to congenic cell transplants [31]. These researchers used improved procedures via the vitelline vessels to inject adult bone marrow into fetal (14 weeks gestation) recipients. Approximately three quarters of all allogeneic recipients lost engraftment by 1 month whereas all congenic recipients maintained permanent multilineage chimerism. These observations clearly show our underestimation of the complexity of the fetal immune system and indicate that more extensive investigations into the mechanism of tolerance or adverse immune reactions to the delivery of xenoproteins *in utero* are warranted.

Undesirable consequences of gene therapy

As reviewed elsewhere, concerns over the safety of gene therapy are intensified in the context of *in utero* application [32,33]. One of these risks, the inadvertent transduction of the male germline, was assessed in sheep, mice and monkeys. Following intraperitoneal injection of a retroviral vector, PCR on purified sperm cells and immunohistochemistry on testis from the born rams revealed low numbers of transduced germ cells [34]. The researchers estimated a transduction frequency of 1 in 6250 germ cells and noted that this was several orders of magnitude below the calculated frequency of naturally occuring endogenous insertions and below the upper tolerable limit set by the FDA [34]. Analysis in mice after intravascular *in utero* injection of HIV [11••] or AAV [35] vector did not detect any germ cell transduction.

Lee *et al* demonstrated transduction of a subset of oocytes after intraperitoneal injection of VSVG pseudotyped HIV GFP vector into late first trimester macaques [36]. Intrapulmonary or intracardiac routes did not result in germline transduction. The researchers suggested that the ontogeny of the female gonads might predispose them to vector transduction, particularly after intraperitoneal delivery early in gestation [36].

Bedrosian *et al* reported transfer of AAV serotypes (2/1,2,5,7,8 and 9) and VSVG pseudotyped HIV delivering GFP cDNA to the fetal murine cochlea [37]. Apart from the technical excellence, this study was noteable in that the AAV-2/1 vector resulted in optimal expression in the inner and outer hair cells of the cochlea several weeks after injection. However, there was also evidence of mild hearing loss in the lentivirus-injected group. This emphasizes the need for careful consideration of vector-related toxicity in fetal gene transfer protocols.

The high level of cellular proliferation in the fetus, the abundance of growth factors and the transcriptionally active state of genes associated with the regulation of growth and

differentiation may predispose the organism to increased risk of cancer from vectors, which integrate into the host genome. Themis *et al* demonstrated a high incidence of hepatocellular carcinoma following delivery of lacZ or human Factor IX cDNA using an equine infectious anemia virus (EIAV) vector, but not after use of an HIV vector [38]. This suggests that the fetus may be uniquely sensitive to genetic perturbations arising from gene transfer, as EIAV has been used in adult animals with no adverse events reported (reviewed in reference [39]).

Although the majority of studies have resulted in long-term expression and immune tolerance (either apparent or experimentally demonstrable), a few have shown immune or inflammatory reactions following *in utero* gene transfer. Jerebtsova *et al* demonstrated production of low titers of antibody against both the vector and the transgenic protein after delivery of both adenovirus and AAV [40]. The strongest response was elicited after adenovirus administration. As mentioned earlier, Seppen *et al* demonstrated long-term reversal of hyperbilirubinemia in Gunn rats [12••], however, in a later publication these researchers reported the detection of antibodies against UDP-glucuronosyltransferase in both mice and rats [41]. In contrast, injection of control GFP vector did not elicit an anti-GFP response and the researchers concluded that UDP-glucuronosyltransferase may be more immunogenic than GFP.

Large animal models

Fetal gene transfer studies on rodents take advantage of the wide range of disease models, short gestation time and the need for relatively small amounts of vector. However, it is important to translate these studies to larger animal models if the goal of human fetal gene therapy is to be achieved. Larger animal models are valuable since they are more relevant to humans in terms of gestation time, maturation of the fetal immune system, and fetal mass as well as in relation to the vector application technology. Fetal mass is an important factor since this will provide data on how to scale vector dosage. Using the sheep model, David *et al* have adapted and further developed minimally invasive ultrasound-guided transcutaneous techniques, as applied in fetal medicine for gene delivery, to several clinically important fetal organ systems, including the liver, airways, gut and musculature [42,43]. These procedures should permit vector administration to the human fetus (reviewed in reference [44]). For example, David *et al* recently investigated gene transfer to the fetal sheep gut by percutaneous injection using ultrasound guidance [43]. An adenovirus vector carrying the β-galactosidase marker gene was injected directly into the fetal sheep stomach in early second trimester, resulting in expression along the entire gastrointestinal tract in four out of eight individuals. The researchers proposed that this might be useful for *in utero* treatment of the early onset gut pathology of cystic fibrosis. Transduction of fetal sheep airways by ultrasound-guided percutaneous intra-tracheal injection [42] or by direct injection, after exteriorization by laparotomy and hysterotomy [45], has also been achieved recently using first generation adenovirus or VSVG pseudotyped HIV, respectively. The percutaneous application achieved a high success rate for gene delivery (33 out of 36 fetuses) with good survival (97%) and a low complication rate (6%).

Tarantal *et al* and Jimenez *et al* have used ultrasound after laparotomy to guide administration of VSVG pseudotyped enhanced GFP (eGFP) expressing HIV vectors to the fetal macaque [46,47]. In one study intrahepatic or intraperitoneal injection was administered toward the end of the first trimester. With both routes, expression lasting up to 7 months in various tissues, particularly those of the abdomen, occurred [46]. In a second study GFP expression was observed in the lung, heart and diaphragm after intrapulmonary and intramyocardial injection at mid-gestation [47].

Other animal and delivery models of *in utero* gene transfer technology

Several papers have described other models of *in utero* gene transfer for studies of fetal gene therapy or for broader application as a research tool in areas such as developmental biology. Two reports describe the administration of VSVG pseudotyped HIV vectors to the fetal rabbit after maternal laparotomy. In the first study vector was injected into the amniotic fluid or, after hysterotomy, into the tracheal lumen between 24 and 26 weeks gestation [48]. In the second study, vector was injected into either the fetal peritoneal cavity or directly into the liver between 20 and 22 weeks gestation [49]. In both studies tissues were harvested at 30 weeks gestation and vector was detected by PCR and immunohistochemistry mainly, but not exclusively, in the target organs. Two recent studies have demonstrated the application of ultrasonography for guidance of vector administration to body compartments of the fetal rat after maternal laparotomy and exteriorization of the uterus. One study demonstrated direct injection of adenovirus and EIAV vectors into the lung parenchyma at 15.5 weeks gestation [50] which resulted in gene expression in interstitial cells. The second study demonstrated injection of HIV and EIAV vectors into the amniotic fluid by ultrasound guidance between 8 and 12 weeks gestation, and by direct visualization between 13 and 18 weeks gestation. The extent and depth of eye structures transduced was inversely proportional to the gestational stage of administration and extensive transduction of the lens and cornea was observed up to 6 months after injection [51]. Two reports describe the use of electroporation to deliver genes specifically as research tools. In the first report, the researchers were able to achieve targeting of a sub-population of cells called interneurons after *in utero* gene transfer to the ganglionic eminence [52]. This was achieved by precise positioning of the electroporation electrodes on the fetal cranium and injection of plasmid into the lateral ventricle. The second report, examining spatial and temporal regulation of gene expression, describes the delivery of plasmids incorporating the Cre/loxP tamoxifen-regulated system for controlled expression of marker genes in the brain [53]. Electroporation was performed at 14.5 weeks gestation and the brains were harvested 2 days after birth. Over the past decade, *in vivo* imaging has become an increasingly powerful research technology. Tarantal *et al* have recently used microPET and whole-body *in vivo* bioimaging after fetal

gene transfer of the marker genes thymidine kinase and firefly luciferase to the fetal macaque [54]. Gene expression was observed for up to 21 months, suggesting that these imaging techniques may provide spatial and temporal data for marker gene expression after *in utero* gene delivery in this nonhuman primate model.

Discussion and conclusion

Several alternatives for the management of genetic disease exist, including symptomatic therapies, pre-implantation diagnosis or termination of pregnancy after prenatal diagnosis [33]. For this reason, as discussed elsewhere, the choice of disease for clinical application requires a high level of certainty that benefit will be provided and that the procedure will not cause additional harm.

The submission of first pre-protocols for human fetal gene therapy in 1998 predated the correction of ADA-SCID and X-linked SCID by stem cell gene therapy and the development of leukemia in three of these patients, two of whom were the youngest to be treated. This resulted in the recommendation that a transduction of bone marrow should be avoided in patients below a certain age, as the characteristics of newborn hematopoiesis might predispose them to insertion in the locus implicated in the onset of leukemia [55]. Therefore, although the ideal of fetal gene therapy is to pre-empt and prevent onset of disease, usually by lifelong expression of a transplanted gene to compensate for a single gene defect, the use of integrating vectors for *in vivo* or *ex vivo* gene therapy will certainly depend on further improvement of their safety features.

In the short term two intermediate strategies for possible *in utero* gene therapy are emerging. The first involves the use of '21st Century' non-integrating vectors, including helper-dependent adenovirus, integration-deficient lentivirus, and new adeno-associated virus serotypes. These may be applied to tissues from which the vector genome is slowly lost, such as muscle [8••], neuronal tissue in the central nervous system [10••], and the retina [9••] for longer-term correction of genetic disease. They may also be applied to rapidly dividing tissues, such as the liver, to permit transient correction of genetic diseases which manifest perinatally until a postnatal treatment can be applied; and may also be applied to the fetus, placenta or extraembryonic membranes for treatment of pregnancy-associated diseases such as pre-eclampsia [27] or bronchopulmonary hypoplasia [18•]. The second and archetypal fetal gene therapy strategy [2,3] is *ex vivo* transduction and subsequent re-implantation. This may now exploit the technological advances in maximizing safety and efficacy of *ex vivo* transduction, novel and safer integrating retroviral vectors, and advances in stem cell technology [29,56].

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• of special interest

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